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ORIGINAL ARTICLE

Influence of sonication process parameters to the state of liquid concentration of extracted rebaudioside A from Stevia (*Stevia rebaudiana bertoni*) leaves

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Abstract The purpose of this work was to develop a process to obtain Stevia extract of a better quality and quantity under influence of ultrasound technique. The chemical compositions, anti-nutritional factor and heavy metals of raw material were tested firstly in (w w^{-1}) in our previous work. The extraction process was assisted by ultrasonication at power 360 W for 12 min using three different types of solvents (water, ethanol and isopropyl alcohol) in different concentrations, that is, 10, 20, 30, 40, 50, and 60% (v v^{-1}) to optimize the extraction process. Stevia leaves extractions, analyzed by HPLC, indicated that isopropyl alcohol (60% v v^{-1}) gave the highest rebaudioside A yield (35 g 100 g^{-1}). This optimum concentration was used in the next set of experiments to optimize ultrasonic power and time. Optimum applied power and sonication time was found 18 min and 480 W, respectively. The extraction yield obtained under optimum process conditions for water, ethanol and isopropyl alcohol were 32.79, 33.85 and 37.10 (g 100 g^{-1}), respectively. Compared to

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classical methods like maceration and heat extraction, the utilization of ultrasound-assisted extraction proved to be a much simpler and efficient way to obtain rebaudioside A from *Stevia rebaudiana* leaves.

1. Introduction

The increasing consumption of sugar (sucrose) has resulted in several nutritional and medical problems, such as obesity. Therefore, low caloric sweeteners have been investigated to substitute sugar. An important class of low caloric sugar substitutes is known as high intensity sweetener, this is at least 50–100 times sweeter than sucrose (Pól et al., 2007). Nowadays, the most common high intensity sweeteners in the world market are made of synthetic compounds. A frequent metallic aftertaste of such synthetic sweeteners does not provide the realistic taste of sugar as well as some types of synthetic sweeteners such as saccharin is associated with the potential risk of cancer of bladder when they are used heavily (Pól et al., 2007). Therefore, low calorie sweeteners are urgently required as substitutes for table sugar. *Stevia rebaudiana* is a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America, e.g. Paraguay and Brazil (Puri et al., 2012). The main sweet component in the leaves of *S. rebaudiana* is stevioside (4–20%, dw) and dulcoside A (0.5%, dw), steviolbioside (trace), and rebaudiosides A (3% dw). The presence of steviolbioside and rebaudioside B in extracts might be due to artifacts of the extraction procedure (Brusick, 2008; Siddique et al., 2013).

The major constituents in the leaves of *S. rebaudiana* are the potently sweet diterpenoid glycosides stevioside, rebaudiosides A and D, and dulcoside A. These compounds, which are known as Stevia sweeteners, are the glycosides of the diterpene steviol, ent-13-hydroxykaur-16-en-19-oic acid (Prakash Chaturvedula et al., 2011). Conventionally, extraction of stevioside from leaves involve two methods that is aqueous or alcohol extraction, followed by precipitation, coagulation and crystallization (Puri et al., 2012). However, modern extraction techniques, such as pressurized liquid extraction, pressurized hot water extraction (PHWE), supercritical fluid extraction and microwave-assisted extraction (MAE), have been used for extracting bioactive compounds (Erkucuk et al., 2009; Prakash Chaturvedula et al., 2011). Recently, enzyme-assisted extraction methods have been also reported to be used in the plant-based bioactive compound extraction (Puri et al., 2012).

Stevia is known for its different steviol glycoside content and its dynamics during ontogeny using HPLC (Erkucuk et al., 2009; Yoda et al., 2003). Kolb et al. (2001) developed an improved HPLC method for quality control of stevioside and rebaudioside A contents in dried leaves of *Stevia rebaudiana*. Geuns et al. (2003) studied metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. The results showed that no traces of stevioside or its contro-versial metabolite steviol were found in the blood of pigs, which is supportive for the safe use of stevioside as a sweetener.

Recently, the application of ultrasound seems to be very promising to obtain the high yield and activities (Li et al., 2007; Wagner, 2013; Wang and Zhang, 2006) as it was concluded from the studies on the extraction of proteins, medicinal compounds, tea solids, carbohydrates, etc. The present work was aimed to develop an alternative ultrasonically assisted extraction procedure that requires less time and minimizes human and environmental exposure. Moreover, optimize the ultrasound-assisted extraction conditions were employed, which could maximize the yield. Besides, the components of the extracts obtained using the optimized processes herein were also compared with that of classical extraction.

2. Experimentals

2.1. Materials

The green Stevia leaves were obtained from the Yancheng Xiaguang Stevioside Trading Company (Jiangsu, China), a high-speed blender (25000/min), type WK – 1000A (Qing Zhou Jing cheng Machinery Co., Ltd., Shandong, China), pH-meter FE20 Mettler-Toledo Instruments (Shanghai, China), Centrifuge CT14D Shanghai Techomp Bio-Equipment Ltd. (Shanghai, China). Sodium hydroxide (NaOH), Sulphuric Acid (H_2SO_4), Hydrochloric acid (HCl), indigo solution, $KMNO_4$, solvents and others chemical were obtained from Sigma Chemical Co. (Shanghai, China). Polymeric adsorbent ADS-7 was obtained from the Tianjin Nankai Hecheng S&T Co., Ltd. (Tianjin, China).

2.2. Ultrasonic-assisted extraction

Dry and ground leaves (10 g) were suspended and extracted in 100 mL of water, under additional stirring (pH value was controlled with 0.01 M pH 7 sodium phosphate. A glass beaker of standard geometry (volume of 100 mL was used) allowed extracting at the same liquid height using ultrasound generator probe, (JY98-III DN, Nanjing Fei qi industry & trade Co., Ltd. Nanjing, China). The actual power delivered into the extraction system was 360 W (at 30% amplitude). An ultrasonic probe with a tip diameter of 20 mm was fitted into a glass beaker and the tip was inserted at the 15 mm height of the extraction solvent. Extraction temperature was controlled by immersing a glass beaker into an automatically adjustable temperature water-bath. After extraction, the crude extracts were centrifuged, filtered and then analyzed by HPLC.

2.3. Ethanol and isopropyl alcohol extraction

Ten gram Stevia leaves powder was soaked in 100 mL a propyl-alcohol (varying propyl-alcohol concentration fraction 10%, 20%, 30%, 40%, 50%, and 60%), and then placed in the ultrasonic extraction equipment and sonicated at 30 °C for certain time (12 min). Sonication with ethanol was done by using the same conditions above. The extracted solution

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was centrifuged and filtered off through 0.45 μm microporous membrane, the filtrate was taken for total rebaudioside A content analysis. The extraction yield of total rebaudioside A content was defined by the HPLC analysis. The highest optimum solvent concentration which give the highest rebaudioside A yield will be taken for further testing to application ultrasonic – assisted power (300, 400 and 480 W) in different time (6,18 and 24 min).

2.4. Transparent solution

Two grams of ferrous sulfate and 1 g of calcium oxide was added to 50 mL of crude extract of Stevia leaves for precipitation. Next, a transparent solution of stevia was obtained by removing the precipitates through filtration.

2.5. Regeneration of ion-exchange resins

Approximately 3 g of macro pores resins were weighed and soaked in 2 volumes of sodium chloride for 22 h then washed with deionized water. The resins was soaked in sodium hydroxide about 2 volumes for 3 h and rewashed with deionized water. 5% 2 volumes hydrochloride for 2 h was added and rewashed by deionized water until neutralize. Soaking the resins with 95% ethanol for overnight then rewashing with deionized water, then the resin was dried at 80 °C until use. The resin physical and chemical properties are presented in Table 1.

2.6. Adsorption resins screening

The adsorption experiments were performed as by Li et al. (2012) with some modification. Pre-weighted amount of MARs (equal to 3 g dry resin) and 50 mL of Stevioside solution were put into conical flasks, with a stopper. Then, the conical flasks were shaken in QYC-2102 incubator Shanghai CIMO Medical Instrument Manufacturing Co., Ltd., (Shanghai, China) at 43.5 °C for 4.5 h. Adsorption experiments were carried out as follows:

After the adsorption equilibrium was reached, the resins were first washed by ultrapure water for six times, and then desorbed with 50 mL ethanol–ethyl acetate solution (3:1; v v^{-1}) in the conical flask in the incubation shaker at 43.5 °C for 0.5 h. The raffinate and desorption solutions were evaporated and dried at 110 °C to constant weight. Thereafter, the remaining part of air dried matter were then dissolved in 25 mL 70% ethanol–water solution and analyzed by HPLC, respectively.

2.7. HPLC analysis

Standard Stevioside, 1 g was accurately weighed and transferred to a 25 mL volumetric flask and the volume was made with water. Solutions of 25, 50, 100, 150, 200, 250, and 300 μg L^{-1} were made by transferring an aliquot from stock solution and the volume was made with water in each case. Further standard solutions were prepared freshly each day by appropriate dilution of stock solution with water for intra-day as well as inter-day analysis.

200 μg L^{-1} of solvent extract was accurately weighed and transferred to a 25 mL volumetric flask and the volume was made by distilled water. Then 10 μL of the stock solution was subjected to HPLC analysis and the concentration of rebaudioside A was calculated based on the calibration curve equation ($y = 36781x + 2887.7$, $R = 0.9993$).

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed and significant difference in mean values were evaluated by Fisher LSD test at ($P < 0.05$) using SPSS version 17.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

Owing to nutritional composition and health-promoting characteristics of Stevia leaves, its phytochemical constituents were studied previously (Gasmalla et al., 2014). Our studies showed that Stevia is a good source of carbohydrates, protein, fiber and minerals.

3.1. Ethanol extraction

As a result, ethanol–water mixture was tested in ultrasonication. The impact of ethanol concentration on the extraction yield of rebaudioside A was demonstrated in Fig. 1a. The extraction yield increases first when ethanol concentration changes from 0% to 30% (v v^{-1}), and goes down when ethanol concentration was above 30%. Therefore, the preferred ethanol concentration was 30% at which the maximum extraction yield was obtained (32 g 100 g^{-1}). Besides the extraction yield obtained using the solvent at 30% ethanol was higher than that using water solvent (30 g 100 g^{-1}). This is probably due to the solvent–solute affinity and the effective swelling of the leaves material by the solvent, which might have increased the surface area for solute–solvent contact.

Table 1 Physical characteristics of polar macroporous ADS-7 resin and optimum sonication process parameters of total rebaudioside A.

Pore diameter (nm)	Bead diameter (mm)	Surface area ($m^2 \cdot g^{-1}$)	Water content (%)
Physical characteristics of polar macroporous ADS-7 resin			
25–30	0.3–1.25	100–150	60–70
Solvents	Sonic power (W)	Time (min)	Rebaudioside A yield (g 100 g^{-1})
Optimum sonication process parameters of total rebaudioside A			
H_2O	360	6	32.79 ± 3.6^{c}
Ethanol 30%	360	6	33.85 ± 1.2^{b}
Isopropanol 60%	480	18	37.10 ± 5.1^{a}

Values are means ± standard deviation of three determinations. Column with different letters indicate statistical differences ($P < 0.05$).

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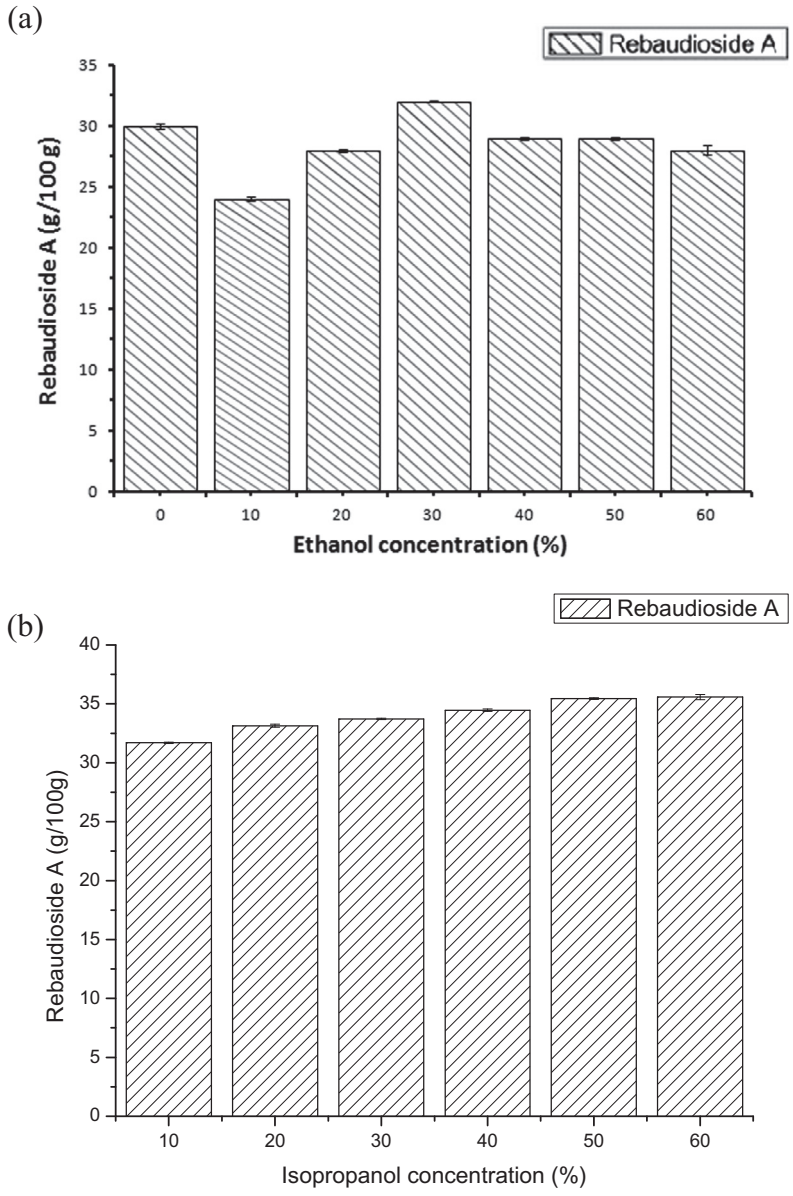

Figure 1 (a) Effect of water and ethanol concentration (%) to determine maximum yield of rebaudioside A from stevia leaves (g 100 g^{-1}). (b) The effect of initial isopropyl alcohol ratio on the extraction yield of total rebaudioside A. 10–60% of propyl-alcohol volume fraction, ultrasonication time: 12 min, extraction temperature: 30 °C.

3.2. Isopropyl alcohol extracts

Ultrasonic power and extraction temperature are two important factors that affect the extraction of total rebaudioside A, the effect of isopropyl alcohol extraction on the extraction yield of total rebaudioside A was showed in Fig. 1b, the extraction yield was increased with the increasing of Isopropyl alcohol ratio using an ultrasonic equipment powered with 360 W. Fig. 1b showed the effect of initial extraction ratio on the extraction yield of total rebaudioside A. The extraction yield was increased fast with the initial extraction isopropyl alcohol ratio increasing from 10% to 60% which was 31 g 100 g^{-1}–35 g 100 g^{-1}, respectively.

It also resulted in the changes of some important physical characteristics of Isopropyl-alcohol rich phase, such as polarity, viscosity and surface tension. This result may have a significant effect on the sonication activity and the partition behavior of rebaudioside A in aqueous system. Where, the extraction yield changes slightly with the change of isopropyl alcohol extraction as shown in Fig. 1b, indicating that extraction ratio has no significant effect on the extraction yield.

3.3. Optimization of ultrasound-assisted of rebaudioside A from stevia leaves

As solvent type water, 30% ethanol and 60% isopropanol were tested to extract rebaudioside A from dried leaves of *Stevia rebaudiana Bert*. Were proved to be beneficial, dissolving the constituents more effectively thus, leading to improvement of the extraction yield as described in Figs. 2 and 3. In general, When the solvent quantity is less than the ratio mentioned

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above, there is not enough liquid to ensure a proper/complete welling of the cell's membrane, leading to a smaller diffusional flux of extraction. Moreover, the concentration of the extracted rebaudioside A into the liquid phase rises faster to the equilibrium value, decreasing this way the extraction driving force. Generally, the extraction efficiency obtained with ultrasonic extraction are 32.79, 33.85 and 37.10 g·100 g^{-1} for water, ethanol and isopropanol, respectively (Table 1) higher than that obtained with maceration extraction which has a beneficial effect as reported by Liu et al. (2010) whom found rebaudioside A is 24.21% with classical extraction based method of preliminary experiments. A 100 g batch of Stevia leaves was extracted in boiling water (1 L) with additional stirring for 2 h. The intensification of the extraction process using ultrasonic fields was considerable, not with the rate of extraction, but with respect to the quantity of extracted rebaudioside A. Due to the highly localized kinetic energy introduced by ultrasounds, the mass transfer across the cell membrane is several times faster and likely the dissipation the extracted species into the bulk of the liquid phase. The same behavior could be noticed when the ultrasonic filed is generated in a bath (ultrasonic assisted batch extraction, Fig. 4. The process was completed after 18 min when we used sonic power 480 W in the case of isopropanol while, the optimum value obtained (32.79 g 100 g^{-1} and 33.85 g 100 g^{-1}) in 6 min with the case of water and ethanol. Increasing sonic power to 400 and 480 W time of extraction at 12, 18 and 24 min would damage the structure of the extracted rebaudioside A, turning it into a biologically unsafe procedure. As expected, the amplitude of the ultrasound waves plays an important role in the extraction intensification, lower amplitudes diminishing the extraction time. From Figs. 3 and 5, we observe that the amplitude effect was asymptotic which means that a tradeoff between the extraction time and the wave amplitude could be found. The way of ultrasounds dissipation into the liquid phase plays also a crucial role as proved by Fig. 4. When using an ultrasonic horn the waves are generated locally in a small volume of the extraction phase and generating a complicated internal circulation which helps the spreading. On the other hand higher values for the time means lower concentrations with detrimental effects upon the separation/purification steps envisaged for the valuable product of the extracted species into the bulk

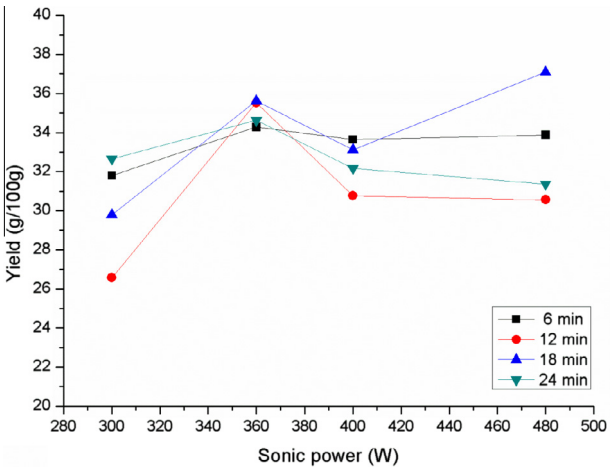

Figure 3 Effects of sonic power on rebaudioside A yield at 30 °C and ultrasound intensity assay, with ultrasound (20 KHz, 300, 360, 400 and 480 W) solvent: isopropanol.

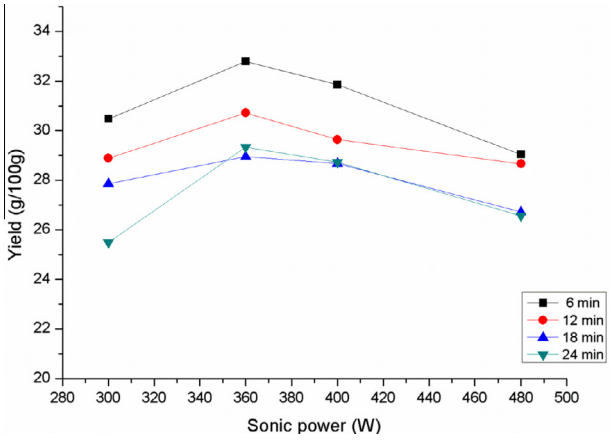

Figure 4 Effects of sonic power on rebaudioside A yield at 30 °C and ultrasound intensity assay, with ultrasound (20 KHz, 300, 360, 400 and 480 W) solvent: water.

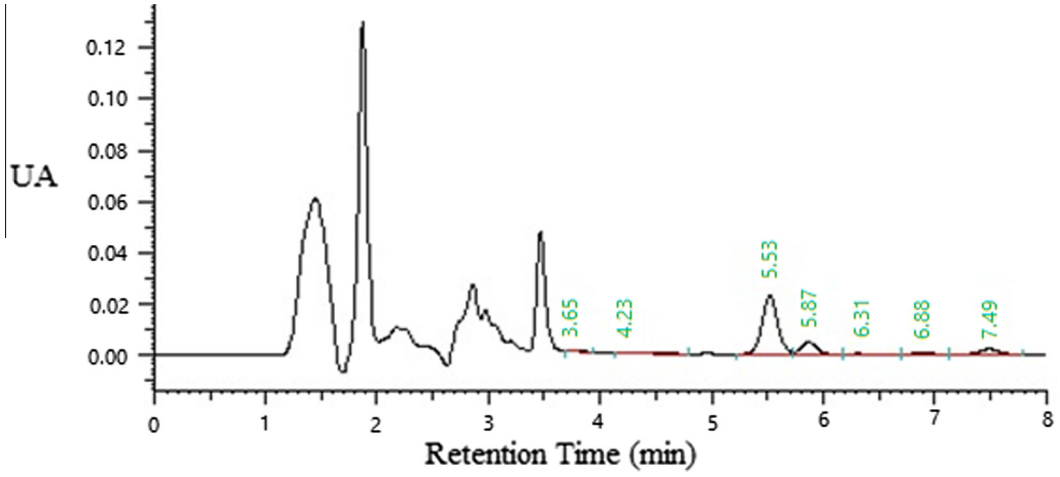

Figure 2 Rebaudioside A peak at 5.53 min retention time.

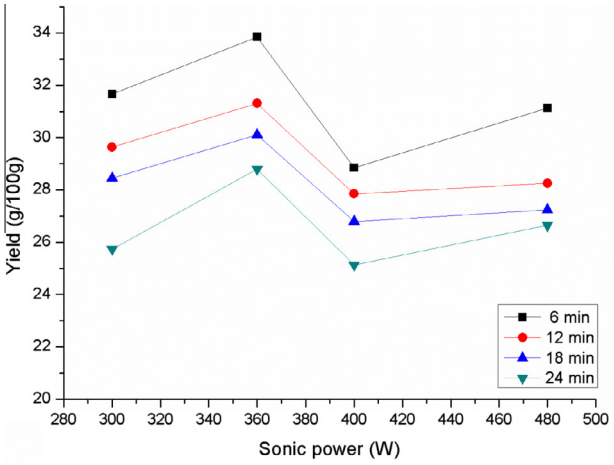

Figure 5 Effects of sonic power on rebaudioside A yield at 30 °C and ultrasound intensity assay, with ultrasound (20 KHz, 300, 360, 400 and 480 W) solvent: ethanol.

liquid. We retained as optimal the sample weight to solvent volume ratio of 60%.

4. Conclusions

In the current study the effects of ultrasound conditions on the extraction efficiency and total rebaudioside A yield were investigated. We found using isopropanol was economically which have higher value (37.10 g/100 g) when we use power 480 W with sonication for 18 min. Also, the presence of water becomes unimportant. The yield in rebaudioside A having a more value as comparing with ethanol 30% which confirmed that the combination of aqueous two-phase separation with ultrasound-assisted extraction improved the extraction of rebaudioside A. Compared with classical methods like maceration and heat extraction, the utilization of ultrasound-assisted extraction proved to be a much simpler and more effective mean to obtain efficiently extractive species from plants. Also, the ultrasound-assisted extraction can be carried out at lower time. This means not only decreasing the operating costs, due to the economy of energy, but also improving the productivity a very important industrial issue. Compared with three solvents the isopropanol proved to be better. More effective ultrasound assisted extraction procedures are needed to obtain rebaudioside A from Stevia plants to work at lower energy consumption and lesser time for the industries.

Acknowledgments

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